

Gene expression profiles of 1-(4-amino-2-methyl-5-pyrimidinyl)-methyl-3-(2-chloroethyl)-3-nitrosourea (ACNU)-resistant C6 rat glioma cells

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Abstract

Chemotherapy in itself is suspected to cause the development or selection of drug-resistant tumor cells, which have more aggressive phenotypes. The authors investigated the differential changes of gene expression in the 1-(4-amino-2-methyl-5-pyrimidinyl)-methyl-3-(2-chloroethyl)-3-nitrosourea (ACNU)-resistant subline of the C6 rat glioma (C6AR2), which was established from C6 rat glioma cells by exposure to ACNU *in vitro*. The resistance to ACNU of C6AR2 was confirmed by MTS assay. The increased expression of O⁶-methylguanine-DNA methyltransferase in C6AR2 cells was shown using RT-PCR. C6AR2 cells displayed a higher proliferative activity relative to C6 cells. Analysis with cDNA array showed that 19 genes were transcriptionally up-regulated and 16 genes down-regulated in C6AR2 cells compared to C6 cells. They belonged to various functional classes of genes beside the drug-resistant system. Among them, the down-regulation of several genes in C6AR2 cells, including c-kit, pleiotrophin, platelet-derived growth factor receptor- α , peripheral myelin protein-22 and NG2 chondroitin sulfate proteoglycan, which are expressed originally in developmental glial lineages, were verified using semi-quantitative RT-PCR. In addition, the gene expression of astroglial

intermediate filament proteins, including GFAP, vimentin and nestin, were decreased in C6AR2 cells relative to C6 cells in semi-quantitative RT-PCR and immunocytochemistry. These findings may represent an undifferentiated state of ACNU-resistant glioma cells and a more aggressive phenotype in recurrent tumors following chemotherapy.

Abbreviations

ACNU (nimustine), 1-(4-amino-2-methyl-5-pyrimidinyl)-methyl-3-(2-chloroethyl)-3-nitrosourea; BCNU (carmustine), 1,3-bis(2-chloroethyl)-1-nitrosourea; GFAP, glial fibrillary acidic protein; MIF, macrophage migration inhibitory factor; MTS, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]; NG2, NG2 chondroitin sulfate proteoglycan; O⁶-MGMT, O(6)-methylguanine-DNA methyltransferase; PDGFR α , platelet-derived growth factor receptor- α ; PMP-22, peripheral myelin protein 22; PTN, pleiotrophin.

Malignant gliomas are the most common type of primary brain tumors. Despite aggressive multimodal treatment, including gross total resection, radiation therapy, and chemotherapy, malignant gliomas are almost uniformly fatal; the median survival time is about one year [1]. Most of these patients die of local tumor recurrence or regrowth, which in most cases is difficult to treat. Although gliomas have a tendency to increase in malignancy during their natural course, recurrent glioma may increase in malignancy compared with the original tumor, possibly in relation to its therapy. Chemotherapy is suspected of having an effect on the generation of phenotypical heterogeneity as well as the development of drug resistance in tumors. Recently, Saito *et al.* [2] reported that 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)-resistant 9L rat glioma cells displayed a more invasive phenotype. Many studies have shown the differential expression changes of drug resistance-related genes in glioma cells acquiring drug resistance [3]. However, there have been few reports about gene expression changes beside drug resistance-related genes [4]. In Japan, 1-(4-amino-2-methyl-5-pyrimidinyl)-methyl-3-(2-chloroethyl)-3-nitrosourea (nimustine hydrochloride, ACNU) is the most common chemotherapeutic agent used in glioma therapy [5,6]. In this study, we developed an ACNU-resistant C6

rat glioma cell line and investigated its differential changes of gene expression.

Materials and Methods

Drugs

ACNU was supplied by Sankyo Drug Co. (Tokyo, Japan). It was solubilized in distilled water and stored at -80 °C until use.

Cells

The C6 rat glioma cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). A C6 subline resistant to ACNU (C6AR2) was established by continuously exposing the cell line to graded concentrations of ACNU. These cells were maintained in Dulbecco's modified Eagle's minimum essential (DME) medium (Sigma, St Louis, MO, USA) supplemented with 10% FBS at 37 °C and 5% CO₂.

Chemosensitivity testing by MTS assay

The sensitivity of C6 and C6AR2 cells was tested using the MTS cell proliferation assay kit (Promega, Madison, WI, USA). C6 and C6AR2 cells in the logarithmic growth phase were plated in 96-well plates in a volume of 100 µl at

1.5 x 10³ cells/well. ACNU was added 16 h later. After incubation for 72 h at 37 °C, the medium was removed from each well and the cells were incubated in a CO₂ incubator for 2 h with 100 µl fresh medium and 20 µl MTS solution, and the absorbance at 490 nm was measured with spectrophotometry. The 50% lethal dose (IC₅₀) for each cell line was determined from the dose-response curves.

Proliferation assay

The proliferative activity of C6 and C6AR2 cells was measured using an MTS assay. C6 and C6AR2 cells in the logarithmic growth phase were plated in 96-well plates in a volume of 100 µl at 0.5 x 10³ cells/well. After incubation for 24, 48 and 72 h at 37 °C, MTS activity was determined as described above.

RNA isolation and cDNA microarray

Total RNA was extracted from cultured glioma cells according to the manufacturer's instructions for TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA). Poly-A⁺ mRNA was isolated and converted to cDNA in the presence of [α-³²P] dATP using an Atlas Pure Total RNA Labeling System according to the manufacturer's protocol (Clontech, Palo Alto, CA, USA). The array membranes

(Rat 1.2k Array, Clontech) were prehybridized for 30 min at 68°C in ExpressHyb, followed by hybridization with the labeled cDNAs overnight at 68°C, washing three times with pre-warmed 2 x saline sodium citrate buffer (SSC) – 1 % sodium dodecylsulfate (SDS) at 68°C for 30 min, once with 0.1 x SSC - 0.5 % SDS for 30 min, and finally with 2 x SSC at room temperature for 5 min. The membranes were immediately wrapped with plastic film, and exposed to a PhosphorImager screen. The screens were scanned using a Molecular Dynamics Storm Phosphorimager and the image data were analyzed with AtlasImage 2.0 software (Clontech). After orientation and alignment of the two array membranes, the array background was subtracted using a local background setting of 200% for all array images (the intensity of spots three-fold greater than the background intensity is significant), and the intensity of the detected genes on each membrane was adjusted by the normalization coefficient, which was calculated based on the intensity of all genes. The data were obtained from the average normalized values of two experiments using AtlasImage. A composite array from duplicate experiments was prepared using AtlasImage software and the ratio of intensity between the different groups was determined. In our study, the ratios were calculated as C6AR2/C6. When the direction of change in signal

levels obtained with two experiments was consistent and the C6AR2/C6 ratio was ≥ 2.0 , it was set as significant up-regulation; if the ratio was ≤ 0.5 , it was set as significant down-regulation. If the signal from one of the arrays was under the background intensity, an intensity difference ≥ 20 (C6AR2 minus C6) was defined as up-regulation and a difference ≤ -20 as down-regulation.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from cultured glioma cells according to the manufacturer's instructions for TRIZOL Reagent. Two μg of total RNA were converted to first strand cDNA using a Reverse Transcription System (Promega). The resulting cDNA was subjected to PCR analysis using a *Taq* PCR Master Mix Kit (Qiagen, Valencia, CA, USA). Each cycle consisted of 30 s at 94°C for denaturation, 30 s at 60°C for annealing, and 60 s at 72°C for extension. A negative control reaction without reverse transcription was also performed. The PCR products were stained with ethidium bromide after agarose gel electrophoresis and photographed using Polaroid film type 667. The intensity of ethidium bromide fluorescence was measured using NIH Image 1.62 program. The cycle number was chosen such that amplification of the products was in the

linear range with respect to the amount of input cDNA. The levels of target mRNAs were normalized to the level of cyclophilin mRNA in the same sample [7,8]. The actual sequences of specific primers are as follows:

O^6 -MGMT	(sense) 5'-ATTAGCAGCCCTGGCAGGCAACCC-3'
	(antisense) 5'-GAAGACTCGAAGGATGGCTTGAGCC-3'
Fas	(sense) 5'-TGCACCAACCTGCCATCCGTGCAC-3'
	(antisense) 5'-CTCAAGGATGTCTTCAAGTCCACACG-3'
MIF	(sense) 5'- CCGGCACAGTACATCGCAGTGCAC-3'
	(antisense) 5'- CCCACGTTGGCTGCGTTCATGTCG-3'
c-kit	(sense) 5'- TGGCGTTTCCTACGTCGTACCAACC-3'
	(antisense) 5'-CTCTCCGGTGCCATCCACTTCACG-3'
PTN	(sense) 5'-CTGTGGAGAATGGCAATGGAGTGTG-3'
	(antisense) 5'-CTGACAGTCGGCATTGTGCAGAGC-3'
PDGFR α	(sense) 5'- GAGCCAGGAGACGAGGTATCAAAGC-3'
	(antisense) 5'-TGCAGATCATCCACTCGATGTTCCGG-3'
PMP-22	(sense) 5'-ACTGTACCACATCCGCCTTGGGAG-3'
	(antisense) 5'- GTCGTTGTTGACATGCCACTCACTG -3'
NG2	(sense) 5'- GTGTTTGACTGTGCACTGGGCTCT-3'

	(antisense) 5'- GGCTTTGGTCCCATCTCAACAAAC-3'
GFAP	(sense) 5'-GCCGCTCCTATGCCTCCTCCGA-3', (antisense) 5'-TCCAGCGACTCAACCTTCCTCT-3'
vimentin	(sense) 5'-GGTGGATCAGCTCACCAATGACAAG-3 (antisense) 5'-GCAAGGATTCCACTTTACGTTCAAGG-3'
nestin	(sense) 5'-AGAGGTGACCCTTGGGTTAGAGGC-3' (antisense) 5'-ATGGACTCAGACTCTTCATGGCCC-3'
cyclophilin	(sense) 5'-GGGGAGAAAGGATTTGGCTA-3' (antisense) 5'-AGAGATTACAGGGTATTGCGAG-3'

Immunocytochemistry

Tumor cells cultured on Lab-Tek chamber slides (Nalge Nunc Internatinal, Rochester, NY, USA) were fixed for 15 min at room temperature in 2% paraformaldehyde. The Envision system (DAKO, Carpinteria, CA, USA) was used for immunocytochemistry. Briefly, the culture slides were washed free of fixative with phosphate-buffered saline (PBS, pH 7.4), exposed to 0.3 % H₂O₂ in PBS for 5 minutes to inactivate endogeneous peroxidase, then incubated in blocking solution (DAKO) for 5 minutes. The slides were then reacted with

primary antibodies for 2 h at room temperature. Dilutions of primary antibodies were 1:1000 for anti-GFAP polyclonal antibody (DAKO), 1:200 for anti-vimentin monoclonal antibody (DAKO), and 1:100 for anti-nestin monoclonal antibody (BD Biosciences, San Jose, CA, USA). Secondary antibodies labelled with horseradish peroxidase (DAKO) were applied to the slides for 1 h. The color was developed with 0.02% 3-3' diaminobenzidine tetrahydrochloride and 0.03% H₂O₂ in PBS. The slides were counterstained with Mayer's hematoxylin. Between steps, the slides were washed three times in PBS. Slides incubated with normal mouse or rabbit serum in place of the primary antibody served as negative controls.

Statistical analysis

Data are expressed as the mean \pm standard error (SEM). Statistical differences were compared between those of C6 and C6AR2 cells using Student's *t*-test. A value of $p < 0.05$ was considered statistically significant.

Results

Sensitivity for ACNU of C6 and C6AR2 cells

Compared to C6 cells, C6AR2 cells showed an increased resistance to ACNU treatment. IC_{50} for ACNU of C6 was $5.0 \pm 0.3 \mu\text{g/ml}$ and that of C6AR2 was $155.9 \pm 12.6 \mu\text{g/ml}$, respectively (Fig.1). Since O^6 -MGMT induction is an important mechanism for resistance to ACNU, we investigated the expression of O^6 -MGMT in C6 and C6AR2 cells using RT-PCR. In C6AR2 cells the expression of O^6 -MGMT was up-regulated relative to C6 cells (Fig.2)

Proliferative activity of C6 and C6AR2 cells

As shown in Fig.3, C6AR2 displayed a higher proliferative activity than C6 cells.

cDNA array analysis

Among 1,176 genes represented on the cDNA array, 397 genes were detected in C6 and 240 genes in C6AR2 cells (Fig.4), with differences in expression levels for 35 genes in C6AR2 cells relative to those in C6 cells

according to our criteria. Out of 35 genes, 19 genes were up-regulated and 16 genes down-regulated in C6AR2 compared to those of C6. Genes were categorized based on their reported or suggested functions and placed into one of 11 broad functional groups (Table 1 and 2) [9].

The direction of change in the mRNA levels of several genes, including Fas, MIF, c-kit, PTN, PMP-22 and PDGFR α , obtained with the RT-PCR results was consistent with that from the cDNA array results (Figs. 4, 5 and 6, Tables 1 and 2).

Down-regulation of several genes in developmental astro- and oligo-glial lineages

Since the cDNA array showed that several genes (c-kit, PTN, PMP-22 and PDGFR α), which are expressed in developmental glial cells, were down-regulated in C6AR2 cells relative to C6 cells, we further studied about additional markers of glial lineages, including NG2, GFAP, vimentin and nestin, using semi-quantitative RT-PCR. The expression of these genes was decreased in C6AR2 cells compared to C6 cells (Fig.6). In addition, immunocytochemistry demonstrated that C6AR2 cells were immunonegative for

GFAP and vimentin, while their immunoreactivity for nestin remained relative (Fig. 7).

Discussion

The chemotherapeutic agent ACNU is one of the most commonly used against malignant gliomas in Japan [5,6]. However, most patients suffer from local recurrence or regrowth of their tumors following ACNU treatment. Furthermore, generally these recurrent tumors have a more aggressive phenotype, which is usually much more difficult to control. Although diverse processes are considered to be involved in the tumor recurrence, chemotherapy in itself may result in the selection or induction of tumor cells with more malignant characteristics. In fact, Saito *et al.* [2] reported (1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU))-resistant 9L rat glioma cells, which were implanted into the rat brain, displayed a more invasive phenotype compared to wild-type 9L cells.

In the present study, to investigate the differential changes of gene expression in C6 glioma cells with the acquisition of resistance to ACNU, we compared gene expression between wild- and ACNU resistant-type of C6 cells, using cDNA array. In addition to the up-regulation of O⁶-MGMT in C6AR2 cells shown by RT-PCR, cDNA array demonstrated that the expression of glutathione

S-transferase π , which is also one of enzymes for resistance to ACNU [3], was increased in C6AR2 cells compared to C6 cells. Beside this drug-resistance related gene, the cDNA array showed us changes of other functional genes in C6AR2 cells. Among them, we noticed that c-kit, PMP-22 and PDGFR α , which are expressed in oligodendroglial progenitor cells [10-14], were down-regulated in C6AR2 cells relative to C6 cells on the cDNA array. The expression of PTN, which is expressed mainly in developmental astroglial cells [15,16], was also decreased in C6AR2 cells compared to C6 cells. We verified these changes using semi-quantitative RT-PCR. Additionally, we showed that the expression of NG2, which is also expressed in oligodendroglial lineages [12,13] and astroglial intermediate filament proteins, including GFAP, vimentin and nestin, was down-regulated in C6AR2 cells compared to C6 cells. These findings suggest that C6AR2 cells express less developmental oligo- and astro-glial markers. In general, more malignant gliomas express less glial markers. Malignant astrocytic tumors are often GFAP negative, and many high-grade gliomas seem to lose GFAP expression [17-20]. GFAP-negative cells proliferate more rapidly than GFAP-positive cells in the same tumor [21,22]. In the present study, GFAP-negative C6AR2 cells also proliferated more rapidly than GFAP-positive

C6 cells. Recently, using laser capture microdissection and cDNA array, Hoelzinger et al. [23] reported that GFAP and vimentin are down-regulated transcriptionally in invasive glioma cells as compared to the tumor core population. These findings suggest that undifferentiated glioma cells induced by chemotherapeutic agents may also display a more aggressive phenotype.

In summary, we found decreased expressions of glial marker genes in ACNU-resistant C6 glioma cells. This finding may represent the undifferentiated state of these cells, although it is not known whether C6 glioma cells are dedifferentiated when they acquire ACNU resistance or whether undifferentiated cells are selected under ACNU exposure, and more aggressive phenotypes in recurrent tumors in clinical cases. In addition, in this study, the cDNA array showed differential expression changes of various functional genes other than drug-resistance and glial marker genes. Although the cellular events from these changes remains unknown, further studies in this field might provide us an attractive choke point for alternative therapies against malignant gliomas.

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Figure Legends

Fig.1 Graph demonstrating the survival of C6 and C6AR2 cells after treatment of ACNU. The surviving fractions were determined by MTS assay, as described in the Materials and Methods. Values are the mean \pm SEM for n=4; the experiment was repeated three times. Where no SEM bar is visible, the SEM was too small to be graphed. C6AR2 cells showed an increased resistance to ACNU exposure relative to C6 cells.

Fig.2 RT-PCR analysis for the O^6 -MGMT gene in C6 and C6AR2 cells. Total RNA was extracted and reverse-transcribed, followed by PCR with specific primers. Cyclophilin served as the unchanging control mRNA. Relative quantitation values of these mRNA levels were normalized with respect to cyclophilin gene expression, and the results of three independent experiments are summarized as a bar graph. Data are expressed as the mean \pm SEM. The expression of O^6 -MGMT gene was increased in C6AR2 cells compared to C6 cells.

Fig.3 Growth curve of C6 and C6AR2 cells. The cell number for each experiment was determined by MTS assay and is shown as a ratio to the number of tumor cells that had been grown in the media. Values are the mean \pm SEM for n=4; the experiment was repeated three times. *Asterisks* denote values significant between C6 and C6AR2 cells at same incubation times ($p<0.05$). Where no SEM bar is visible, the SEM was too small to be graphed. C6AR2 cells proliferated more rapidly than C6 cells.

Fig.4 Phosphoimages of the expression pattern of genes in C6 (A, top) and C6AR2 (B, bottom) cells. Differential hybridization of two identical Atlas Rat 1.2 cDNA expression Arrays was carried out. This is a representative result from one of two experiments. Arrows (A) and close-up images (B) indicate several genes differentially expressed in glioma cells: 1, Fas; 2, MIF; 3, c-kit; 4, PTN; 5, PMP-22; 6, PDGFR α .

Fig.5 Verification of cDNA array results with semi-quantitative RT-PCR. Two up-regulated genes, Fas and MIF, in C6AR2 cells and two down-regulated genes, c-kit and PTN, in those were selected from cDNA array results.

Cyclophilin was used as the unchanging mRNA. Relative quantitation values of these mRNA levels were normalized with respect to cyclophilin gene expression, and the results of three independent experiments are summarized as a bar graph. Data are expressed as the mean \pm SEM. *Asterisks* denote values significant between those of C6 and C6AR2 cells ($p < 0.05$). The direction of change in mRNA levels obtained with the RT-PCR results was consistent with that from the cDNA array results.

Fig.6 RT-PCR analysis for genes expressed in developmental oligo- and astro-glial cells, including PMP-22, PDGFR α , NG2, GFAP, vimentin and nestin. Total RNA was extracted and reverse-transcribed, followed by PCR with specific primers. Cyclophilin served as the unchanging control mRNA. Relative quantitation values of these mRNA levels were normalized with respect to cyclophilin gene expression, and the results of three independent experiments are summarized as a bar graph. Data are expressed as the mean \pm SEM. *Asterisks* denote values significant between those of C6 and C6AR2 cells ($p < 0.05$). Where no SEM bar is visible, the SEM was too small to be graphed. The gene expression of glial markers was down-regulated in C6AR2 cells

relative to C6 cells.

Fig.7 Photographs of immunocytochemistry for astroglial intermediate filament proteins (A and D, GFAP; B and E, vimentin; C and F, nestin) in C6 (A, B and C) and C6AR2 (D, E and F) cells. Bars represent the length of 50 μ m. Immunoreactivity for GFAP and vimentin was lost in C6AR2 cells, while that for nestin remained relatively.

Table 1. Genes up-regulated in C6AR2

Gene Families and Names	GenBank	Ratio
<u><i>Apoptosis, DNA-binding and cell cycle-regulating proteins</i></u>		
fas antigen (APO-1)	D26112	2.0
G1/S-specific cyclin D3 (CCND3)	D16309	2.0
LIM domain protein CLP36	U23769	2.5
<u><i>Hormone, neurotransmitters and receptors</i></u>		
inositol triphosphate receptor subtype 3	L06096	2.3
insulin receptor	M29014	2.0
<u><i>Immune system proteins</i></u>		
macrophage migration inhibitory factor (MIF)	U62326	2.0
<u><i>Metabolism</i></u>		
glutathione S-transferase P subunit	X02904	2.0
glutathione S-transferase Yb subunit	J02592	3.0
glutathione transferase subunit 8	X62660	2.0
glycerol kinase	D16102	2.5
squalene epoxidase	D37920	2.0
<u><i>Proteases and protease inhibitors</i></u>		
amonipectidase B	U61696	2.0
<u><i>Signal transduction</i></u>		
beta-arrestin 1	M91589	2.5
casein kinase II alpha subunit	L15618	2.0
NVP-3 (neural visinin-like protein 3)	D13126	2.1
protein kinase C-zeta	M18332	2.0
<u><i>Translation and protein turn-over</i></u>		
proteosome component C13	D10729	2.0
<u><i>Transporters and carrier proteins</i></u>		

Insulin-like growth factor binding protein-1		
(IGFBP1)	M89791	2.2
Na ⁺ /K ⁺ ATPase beta 3 subunit	D84450	4.9

Genes up-regulated in C6AR2 cells relative to C6 cells. Data were obtained from quantification of cDNA arrays using the Atlas Image software after normalization. Gene expression was calculated by determining the average of the ratio of C6AR2/C6 from the results of duplicate experiments.

Table 2. Genes down-regulated in C6AR2

Gene Families and Names	GenBank	Ratio
<u><i>Adhesion molecules, cytoskeletons and ECMs</i></u>		
peripheral myelin protein 22 (PMP-22)	M69139	0.2
<u><i>Apoptosis, DNA-binding and cell cycle-regulating proteins</i></u>		
G1/S-specific cyclin D1 (CCND1)	D14014	0.3
high mobility protein 2 (HMG2)	D84418	0.4
<u><i>Growth factors and growth factor receptors</i></u>		
c-kit		0.5
PDGF receptor alpha (PDGFR α)	M63837	0.5
pleiotrophin (PTN)	M55601	Down -74*
<u><i>Metabolism</i></u>		
cytochrome c oxidase Vb & VIa precursor	D10592+X14208	0.3
mitochondrial ATPase synthase beta subunit	M19044	0.5
<u><i>Proteases and protease inhibitors</i></u>		
tissue carboxypeptidase inhibitor (TCI)	U40260	0.2
<u><i>Signal transduction</i></u>		
14-3-3 protein eta (PKC inhibitor protein-1)	D17445	0.5
PDGF-associated protein	U41744	0.4
<u><i>Transcription</i></u>		
DNA-binding protein inhibitor ID-1	D10862	0.4
DNA-binding protein inhibitor ID-3	D10864	0.5
<u><i>Translation and protein turn-over</i></u>		
proteasome subunit RC10-II	D21800	0.5
<u><i>Transporters and carrier proteins</i></u>		
epidermal fatty acid binding protein (E-FABP)	U13253	Down -21*

Na ⁺ /K ⁺ ATPase alpha1 subunit	M28647	0.5
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Genes down-regulated in C6AR2 cells relative to C6 cells. Data were obtained from quantification of cDNA arrays using the Atlas Image software after normalization. Gene expression was calculated by determining the average of the ratio of C6AR2/C6 from the results of duplicate experiments.

* Down : Difference between signal intensity of C6AR2 and C6 (a ratio could not be calculated because the signal of C6AR2 was below background).

Fig.1

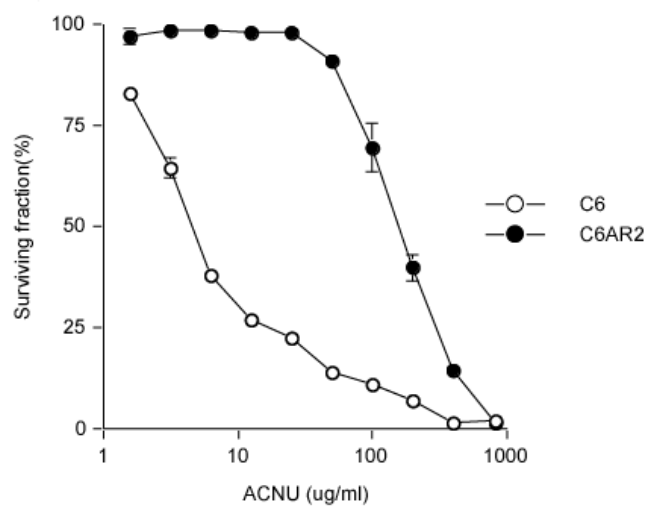


Fig.2

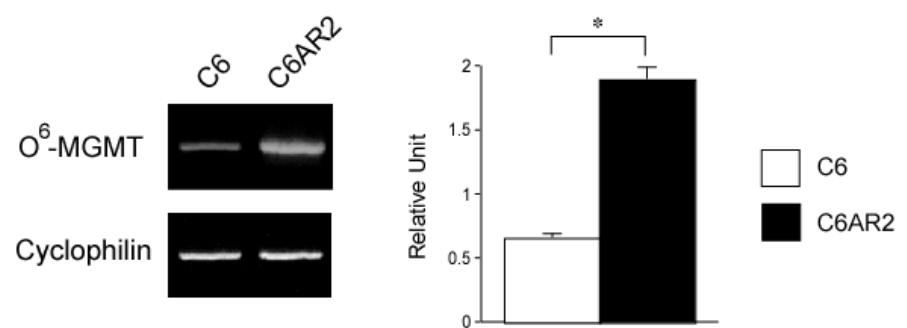


Fig.3

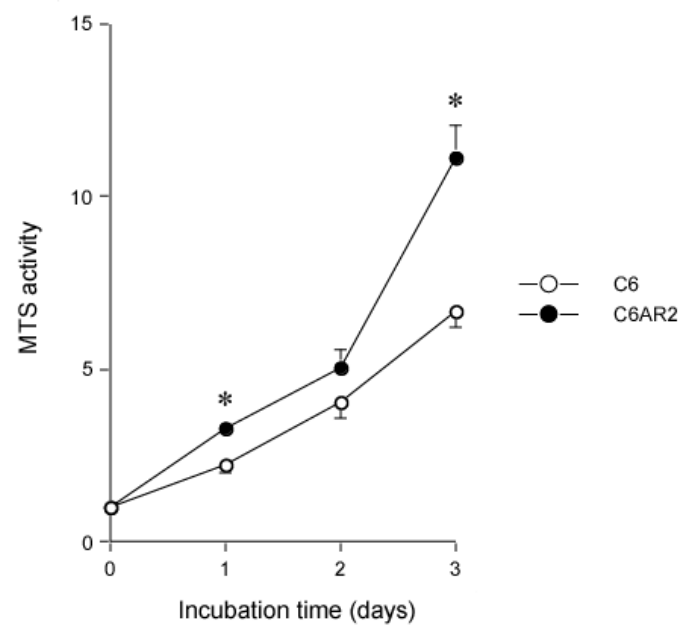


Fig.4

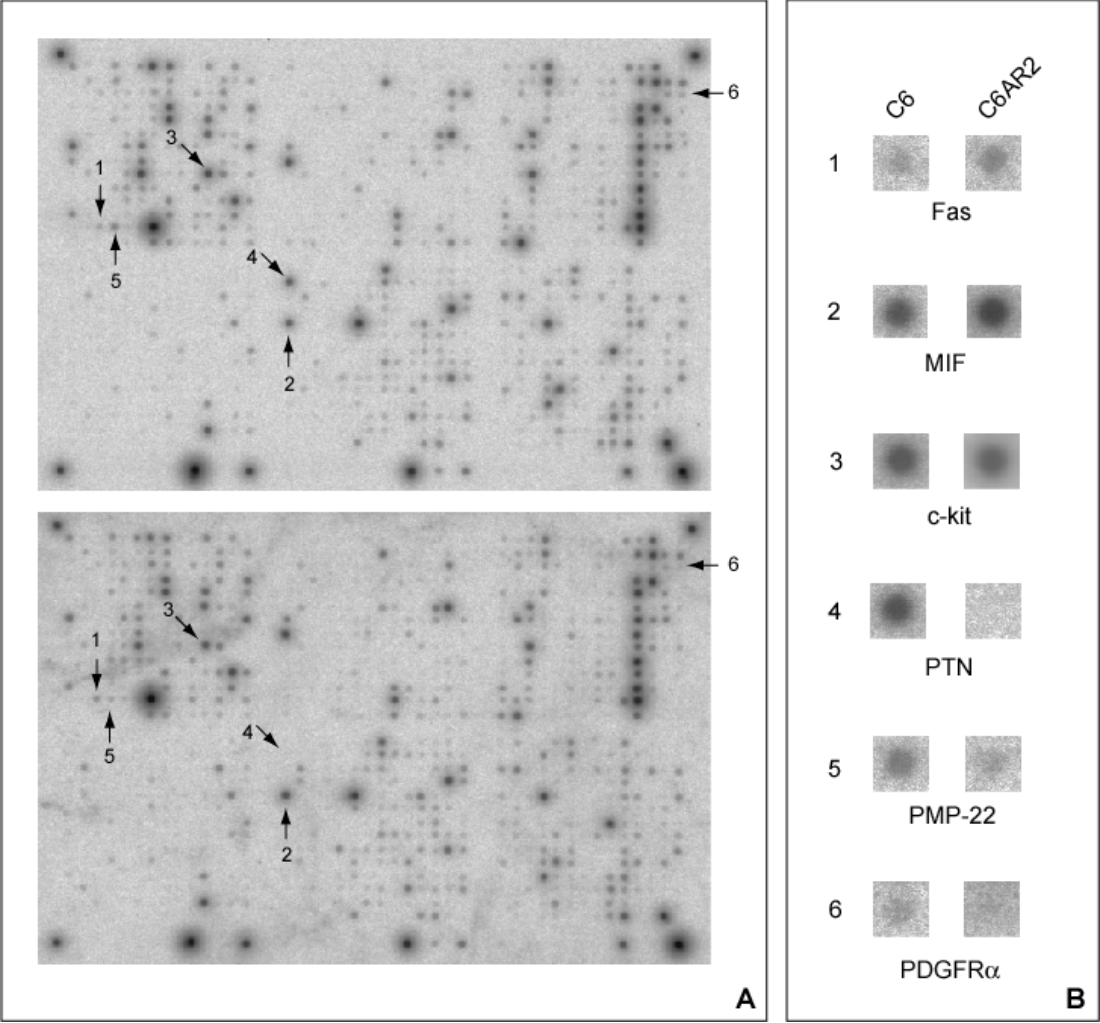


Fig.5

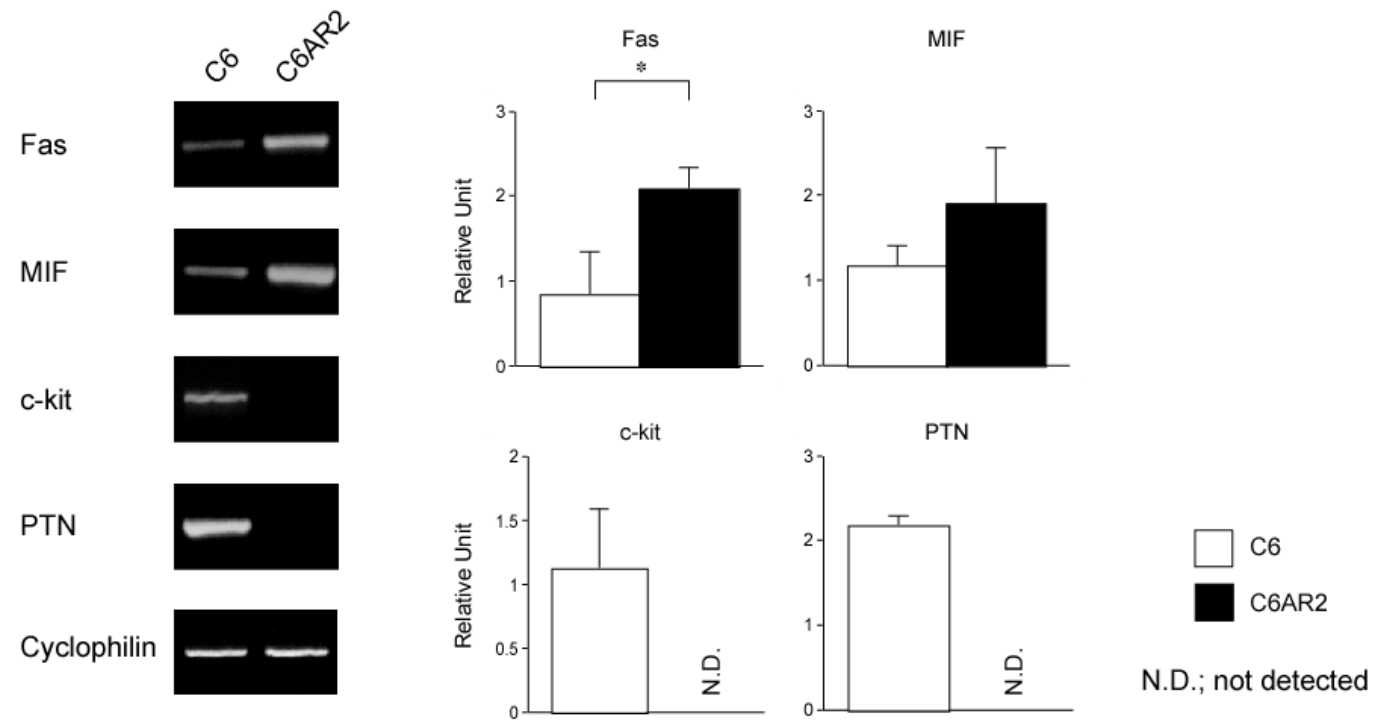


Fig.6

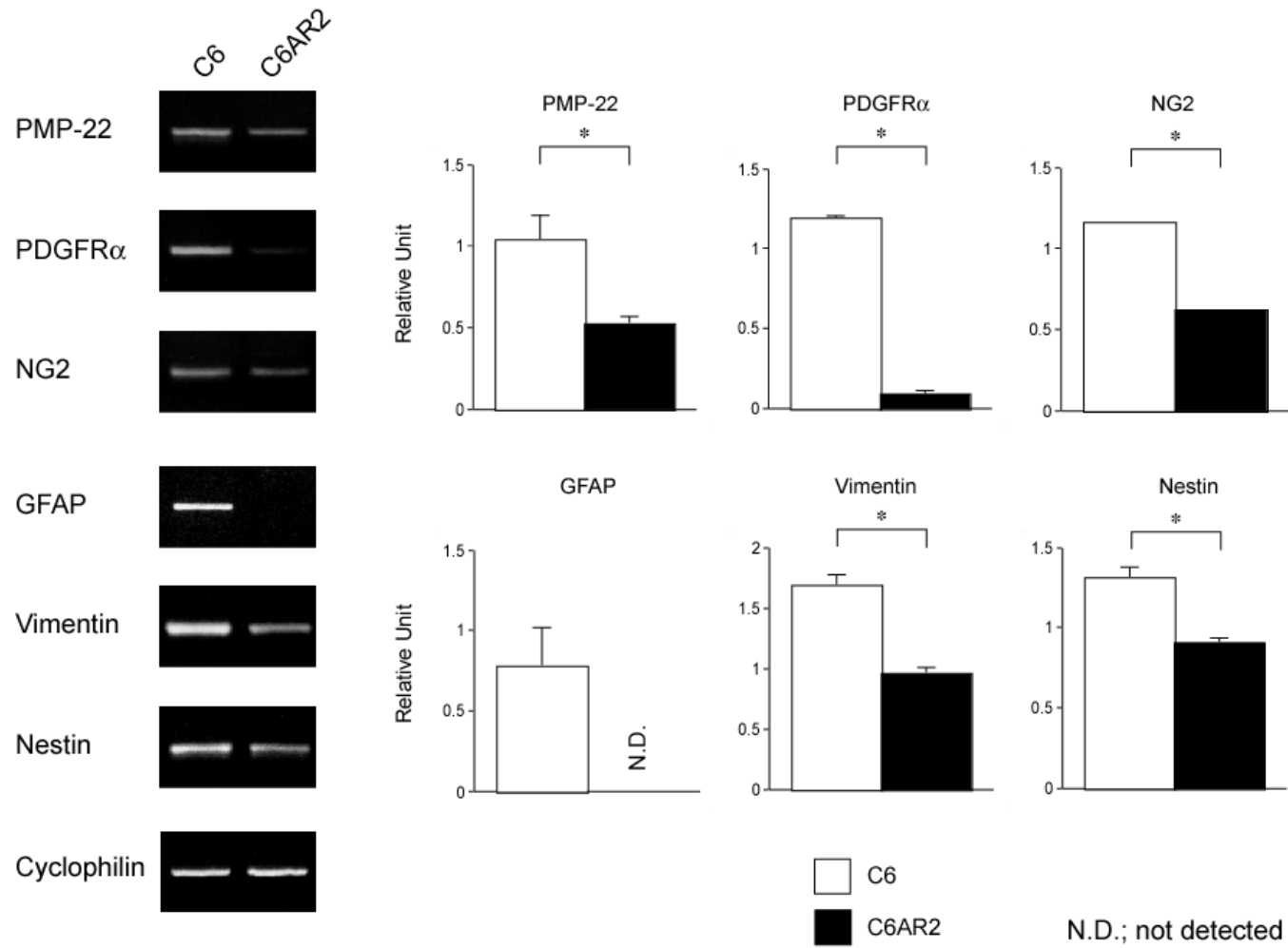


Fig.7

